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Granulocyte Colony-Stimulating Factor and Its Receptor in Acute Promyelocytic Leukemia

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Expression of granulocyte colony-stimulating factor (G-CSF) receptor (G-CSFR) and in vitro proliferative response to G-CSF were investigated by quantitative immunofluorescence and [^{3}H] thymidine uptake, respectively, in a series of acute myeloid leukemias (AML). The results indicated that G-CSFR was detected at high levels in acute promyelocytic leukemia (APL) cells, in comparison with other types of AML. Moreover, APL cells were also seen to predominantly proliferate in response to G-CSF. Based on these observations, we administered recombinant human G-CSF to a patient with APL in the third relapse that was resistant to both cytotoxic agents and all trans retinoic acid, in an attempt to sensitize the leukemic cells to cell-cycle-dependent agents. Complete remission was achieved. The finding that APL cells are exquisitely responsive to G-CSF supports the view that G-CSF is useful for augmentation of their vulnerability to cell-cycle specific agents. Am. J. Hematol. 58:31–35, 1998. © 1998 Wiley-Liss, Inc.

Key words: G-CSF; G-CSF receptors; acute myeloid leukemia; acute promyelocytic leukemia; cell-cycle active drugs

INTRODUCTION

Hematopoietic growth factors are implicated in various clinical entities [1,2]. Granulocyte colony-stimulating factor (G-CSF) was administered to patients with acute myeloid leukemia (AML) to enhance sensitivity of AML cells to cytotoxic drugs as well as to shorten the period of chemotherapy-induced neutropenia [1,3–5]. Although G-CSF indeed shortened neutropenia after combined chemotherapy in patients with AML, the potential of G-CSF to increase cytotoxicity of cell-cycle-specific drugs varied with the patients.

Conventional chemotherapy with cytotoxic agents and differentiation therapy with all-trans-retinoic acid (ATRA) can lead to a high incidence of clinical remission in patients with acute promyelocytic leukemia (APL) [6–8]. Patients who have acquired resistance to both cytotoxic and differentiation therapies require much further attention. In our screening for expression of the G-CSF receptor (G-CSFR) and for proliferative effects of G-CSF on AML cells, G-CSFR was highly expressed on APL cells and the proliferative response of APL cells to G-CSF was evident in comparison with that of leukemic cells from other types of AML. Based on our in vitro observations, we treated a patient with APL in the third relapse that was resistant to both cytotoxic agents and ATRA, with the simultaneous administration of cell-cycle active drugs and G-CSF.

MATERIALS AND METHODS

Patients and Leukemic Cells

Leukemic samples were obtained from 81 Japanese adult patients with AML. All patients gave informed con-

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TABLE I. Expression of G-CSF Receptor on AML Cells†

FAB classification	Mean ± SD of RFI	No. of cases: Total	RFI: <1	1–5	5–10	10–15	15–20	>20
M1	5.7 ± 5.1*	22	1	11	5	3	2	
M2	5.7 ± 4.7*	27		17	5	3	2	
M3	31.7 ± 12.6	10					1	9
M4	5.6 ± 2.7*	11		5	6			
M5	5.1 ± 3.4*	11		7	3	1		

†Expression of G-CSF receptor on AML cells was analyzed by quantitative flow cytometry. The values of RFI were estimated as described in Materials and Methods. Data represent mean ± SD.
*Differences from the RFI of M3 are significant at $P < 0.001$.

sent. The diagnosis was made according to the French-American-British (FAB) classification [9]. Leukemic cells were isolated by Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and were cryopreserved at −80°C in fetal calf serum (FCS) Hyclone Laboratories Inc., Logan, UT) with 10% dimethysulfoxide, then stored in liquid nitrogen until use.

Flow Cytometric Detection of G-CSF Receptors

Expression of G-CSFR was analyzed by immunofluorescence using a flow cytometer (Cytron Absolute, Ortho-Clinical Diagnostic Inc., Tokyo, Japan). AML cells were first incubated with anti-human G-CSFR monoclonal antibody (MoAb) (mouse immunoglobulin G_1) (PharMingen, San Diego, CA) for 45 min on ice, washed with cold Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS), and then incubated for 30 min on ice with fluorescence isothiocyanate (FITC)-conjugated sheep anti-mouse IgGs (Cappel, Durham, NC). After washing with cold PBS, the cells were analyzed. Purified mouse IgG_1 (Zymed Laboratories Inc., San Francisco, CA) was used as an isotype control. G-CSFR on AML cells was quantitatively detected by estimating relative fluorescence index (RFI). The RFI values referred to a gated leukemic cell population set on scatter properties. When x and y are channel numbers of test samples and control samples, respectively, RFI is given by the following equation according to the manufacturer's recommendations: $RFI = 10^{3.5(x-y)/255}$ [10].

Cell Proliferation Assay

Purified recombinant human G-CSF (rhG-CSF) was provided by Kirin Brewery Co. Ltd., Tokyo, Japan. To assess proliferation of AML cells, the tritiated thymidine ([^{3}H]TdR) uptake assay was used, using a modification of the method of Griffin et al. [11]. Triplicate aliquots of 2×10^4 cells were suspended in 96-well microplate in 100 μl of RPMI 1640 medium supplemented with 10% FCS in the presence or absence of rhG-CSF and cultured at 37°C in a humidified atmosphere flushed with 5% CO_2 for 2 days. Six hours before cell harvest, 0.1 μCi of [^{3}H]TdR was added to each well. The cells were harvested onto nitrocellulose filters using an automatic cell harvester and the radioactivity was measured in a scintillation counter. The proliferation of AML cells with G-CSF was expressed as stimulation index (SI). SI was calculated as the mean value of radioactivity with G-CSF minus the mean value of radioactivity without G-CSF and divided by the mean value of radioactivity with G-CSF in each AML case.

Statistical Analysis

Statistical analyses were made using a *t*-test.

RESULTS

G-CSF Receptors on AML Cells

G-CSFR on AML cells from 81 patients was quantitatively measured by RFI. The results clearly showed that the level of G-CSFR expression in APL (M3) cells was generally higher than seen in other types of AML (Table I). The mean RFI value of APL cells was more than fivefold compared with findings obtained with other types of AML cells.

Stimulation of AML Cells by G-CSF

The assay for [^{3}H]TdR uptake was limited to 34 patients. We compared the stimulatory effects of G-CSF on APL cells with effects on other types of AML cells (Table II). G-CSF strongly stimulated [^{3}H]TdR uptake of APL cells. The mean SI of APL (M3) was 15.3 and, in all APL cases, the SI exceeded 5. In contrast to results obtained with APL, the SI of the other subtypes of AML was less than 5, except for one M1 case.

CASE REPORT

A 44-year-old Japanese woman was diagnosed with APL in July 1991. The chromosome abnormality, t(15;17), was detected in the bone marrow. She was

TABLE II. G-CSF Stimulation of [^{3}H] Thymidine Uptake by AML Cells†

FAB classification	Mean ± SD of stimulation index	No. of cases						
			Stimulation index					
		Total	<1	1–2	2–3	3–5	5–10	>10
M1	2.3 ± 2.7*	10	2	4	2	1	1	
M2	1.4 ± 1.2*	13	7	4		2		
M3	15.3 ± 12.2	5					2	3
M4	1.4 ± 1.5*	2	1		1			
M5	1.7 ± 1.9*	4	1		2			

†A total of 2×10^4 AML cells were cultured in 96-well microtiter plates. After 2 days, 0.1 μCi/well of [^{3}H] thymidine was added to each well for the last 6 hr of the incubation. [^{3}H] thymidine uptake was measured using a scintillation counter. Stimulation index was calculated as [radioactivity of cells cultured with G-CSF] − [radioactivity of cells cultured without G-CSF]/[radioactivity of cells cultured without G-CSF]. Data represent mean ± SD.

*Differences from the stimulation index of M3 are significant at $P < 0.001$.

treated with combined chemotherapy including behenoylcytosine arabinoside, daunorubicin, and 6-mercaptopurine (BHAC-DM regimen), and achieved complete remission (CR). After a leukemia-free period of 18 months, she had a relapse in February 1993. The patient was given ATRA to induce CR and achieved second CR. In January 1994, the second relapse occurred. She failed to achieve CR by treatment with the BHAC-DM regimen or ATRA. We administered cytosine arabinoside (Ara-C) in a dose of 120 mg per day by continuous intravenous infusion and aclarubicin hydrochloride (ACR) in a dose of 20 mg per day by drip infusion for 7 days. She achieved CR for the third time and was given three courses of the same therapy. After a leukemia-free period of 6 months, the third relapse occurred in November 1994 (Fig. 1). We then treated this patient with Ara-C plus ACR, but CR was not achieved. Bone marrow study showed hypercellularity with 69.2% pathological cells. Karyotype abnormality t(15;17) was evident in the bone marrow. In peripheral blood, the white blood cell count was $1.4 \times 10^3/\mu l$ with 13% pathological cells, hemoglobin was 8.5 g/dl, and platelet count was $37 \times 10^3/\mu l$. The same chemotherapy, Ara-C plus ACR, was repeated, but the outcome was not favorable. In view of our observation that APL cells highly express G-CSFR and respond to G-CSF, we attempted to induce CR by simultaneous administration of rhG-CSF (filgrastim, Kirin Brewery Co., Ltd., Sankyo Co., Ltd., Tokyo, Japan) in a dose of 300 μg per day under the identical regimen consisting of Ara-C and ACR. The white blood cell count was $1.4 \times 10^3/\mu l$ with 51% pathological cells, hemoglobin was 7.4 g/dl, and platelet count was $13 \times 10^3/\mu l$ before this G-CSF-combined chemotherapy. The white blood cell count continued to be less than $0.5 \times 10^3/\mu l$ for 2 weeks following this therapy. This patient had also required platelet and red cell transfusions within approximately 4 weeks after initiation of this G-CSF-combined chemotherapy. Bone marrow before the discontinuation of G-CSF administration was hypocellular, in association with a significant reduction in pathological cells. The karyotype in all metaphases so far examined was normal. The patient underwent another two courses of the G-CSF-combined chemotherapy. Bone marrow examination after the second therapy showed absence of pathological cells with a normal karyotype. The patient remained in CR with a leukemia-free period of 10 months. She died of cerebral hemorrhage during CR. This therapy was well tolerated.

DISCUSSION

We analyzed the quantitative expression of G-CSFR on AML cells using RFI. APL cells expressed high levels of G-CSFR compared with findings in leukemic cells obtained from other types of AML. APL cells also responded to G-CSF more exquisitely than did leukemic cells from other types of AML. Based on these in vitro findings, we anticipated that G-CSF would predispose APL cells to enter into active cell-cycling, which could lead to susceptibility to cell-cycle active agents. The leukemic cells from this patient were not examined for the expression of G-CSFR or the response to G-CSF. We treated a patient with refractory APL with the simultaneous administration of rhG-CSF in combination of Ara-C plus ACR therapy. This patient received two courses of the same Ara-C plus ACR therapy before use of rhG-CSF; however, there was an increase in the proportion of leukemic cells within the bone marrow and peripheral blood following two courses of the Ara-C plus ACR therapy. The patient achieved CR with the administration of rhG-CSF during and after the identical Ara-C plus ACR therapy. It is conceivable that the CR was attributed to the simultaneous administration of rhG-CSF during Ara-C plus ACR therapy. Concerning the mechanism by which the G-CSF-combined chemotherapy elicited CR in our patient, it is possible that G-CSF induced

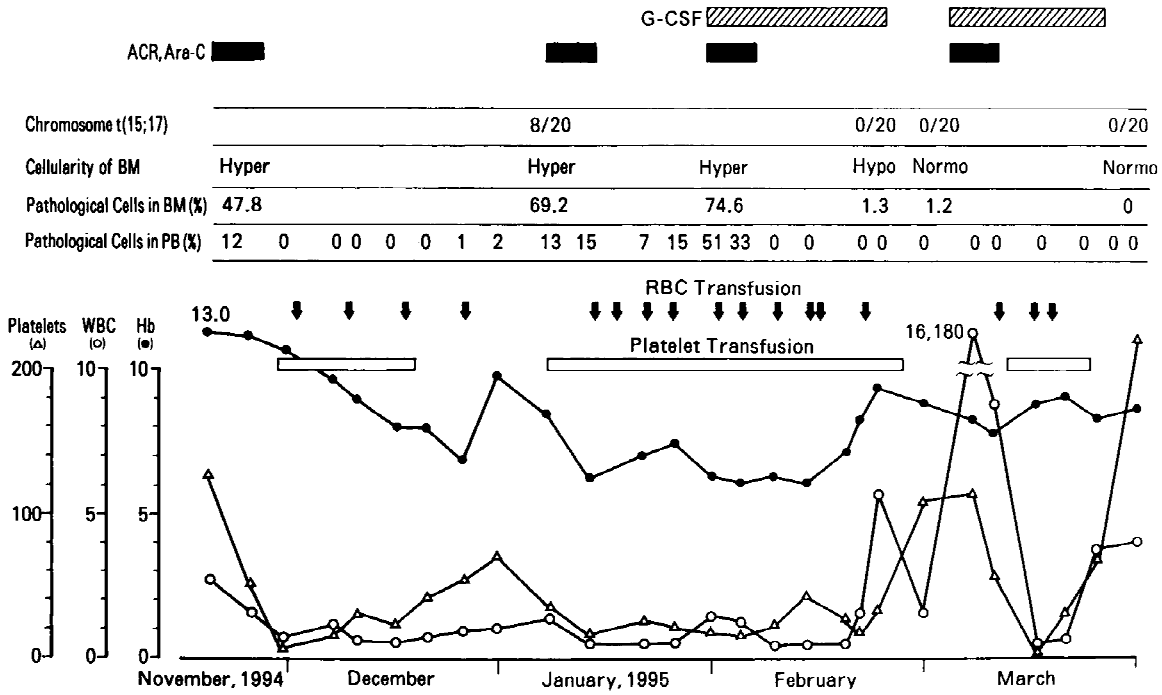

Fig. 1. Clinical course of the patient. ACR, aclarubicin; Ara-C, cytosine arabinoside; G-CSF, granulocyte colony-stimulating factor; BM, bone marrow; Hyper, hypercellular; Hypo, hypocellular; Normo, normocellular; PB, peripheral blood; WBC, the white blood cell count; Hb, hemoglobin. WBC, ×10³/μl; Hb, g/dl; Platelets, ×10³/μl.

refractory APL cells to differentiate into mature polymorphonuclear cells [12–14]. However, we observed a definite cytoreduction phase in the bone marrow as well as in peripheral blood before hematopoiesis recovered. Thus, it is suggested that G-CSF augmented the cytotoxic effects of Ara-C plus ACR on refractory APL cells.

Although use of G-CSF before and during chemotherapy for AML, except for APL, was found to be not beneficial, little is known of the in vivo effects of G-CSF regarding treatment of APL [3–5]. It is likely that G-CSF has not been administered to patients with APL, since in vitro and in vivo studies [15–17] suggested that APL cells would expand in response to G-CSF. We assumed that the induction of entry into the cell-cycle was essential to eradicate refractory APL cells. Although administration of G-CSF during chemotherapy may possibly increase the chemosensitivity of normal hematopoietic stem cells and a long duration of myelosuppression would ensue, the myelosuppression in our patient was not profound. The administration of G-CSF during cytotoxic chemotherapy may provide one therapeutic approach for refractory APL but not untreated APL or other types of AML.

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